

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification:</b> C12N 15/12, A61K 38/17, C07K 14/705, G01N 33/68	<b>A1</b>	<b>(11) International Publication Number:</b> WO 00/68377 <b>(43) International Publication Date:</b> 16 November 2000 (16.11.2000)
<b>(21) International Application Number:</b> PCT/EP00/03683 <b>(22) International Filing Date:</b> 25 April 2000 (25.04.2000) <b>(30) Priority Data:</b> 99109094.5 07 May 1999 (07.05.1999) EP <b>(60) Parent Application or Grant</b> MERCK PATENT GMBH [/]; (). CLEMETSON, Kenneth, J. [/]; (). CLEMETSON, Kenneth, J. [/]; (). MERCK PATENT GMBH; ().	<b>Published</b>	
<b>(54) Title:</b> RECOMBINANT PLATELET COLLAGEN RECEPTOR GLYCOPROTEIN VI AND ITS PHARMACEUTICAL USE <b>(54) Titre:</b> GLYCOPROTEINE VI DE RECONSTRUCTION DU RECEPTEUR DE COLLAGENE DES PLAQUETTES ET UTILISATION PHARMACEUTIQUE DE CETTE DERNIERE		
<b>(57) Abstract</b> <p>The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit binding of thrombocytes and platelets, respectively, to collagen. Changes in GPVI can be used to monitor platelet age and exposure to thrombotic and cardiovascular diseases.</p> <b>(57) Abrégé</b> <p>L'invention concerne la glycoprotéine VI (GPVI), son isolation, sa purification et des procédés de production par reconstruction. En particulier, l'invention a pour objet l'utilisation de la GPVI, de préférence la GPVI de reconstruction, dans le traitement de troubles et de problèmes pathologiques liés directement ou indirectement aux troubles de la coagulation sanguine, telles que les maladies cardiovasculaires et les thromboses. La protéine de reconstruction extracellulaire peut également être utilisée dans des dosages pour détecter des inhibiteurs potentiels de la GPVI liée à la membrane pour inhiber la liaison des thrombocytes et des plaquettes, respectivement, au collagène. Les modifications de la GPVI permettent de contrôler l'âge des plaquettes et l'exposition aux maladies cardiovasculaires et aux thromboses.</p>		

ATTORNEY

ATTORNEY DOCKET NUMBER: 7853-211

SERIAL NUMBER: 09/610,118

REFERENCE: AE

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : C12N 15/12, C07K 14/705, A61K 38/17, G01N 33/68		A1	(11) International Publication Number: WO 00/68377 (43) International Publication Date: 16 November 2000 (16.11.00)
(21) International Application Number: PCT/EP00/03683 (22) International Filing Date: 25 April 2000 (25.04.00) (30) Priority Data: 99109094.5          7 May 1999 (07.05.99)          EP (71) Applicant (for all designated States except US): MERCK PATENT GMBH [DE/DE]; Frankfurter Str. 250, D-64293 Darmstadt (DE). (72) Inventor; and (75) Inventor/Applicant (for US only): CLEMETSON, Kenneth, J. [GB/CH]; Fluracker 29, CH-3065 Bern (CH). (74) Common Representative: MERCK PATENT GMBH; D-64271 Darmstadt (DE).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  Published With international search report.	
(54) Title: RECOMBINANT PLATELET COLLAGEN RECEPTOR GLYCOPROTEIN VI AND ITS PHARMACEUTICAL USE (57) Abstract <p>The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit binding of thrombocytes and platelets, respectively, to collagen. Changes in GPIV can be used to monitor platelet age and exposure to thrombotic and cardiovascular diseases.</p>			

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**Description**

5

10

15

20

25

30

35

40

45

50

55

## RECOMBINANT PLATELET COLLAGEN RECEPTOR GLYCOPROTEIN VI AND ITS PHARMACEUTICAL USE

The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit interaction of platelets and collagen. GPVI on the platelet surface is modified during the platelet lifetime in vivo and can therefore be used as a marker of the platelet age profile.

Glycoprotein VI is a 62/65 kDa (non-reduced/reduced respectively) platelet membrane glycoprotein which forms a complex together with the Fc $\gamma$  common subunit. The GPVI subunit contains the collagen binding site and the Fc $\gamma$  subunit is responsible for signalling. The complex forms one of the major collagen receptors on the platelet surface, critical for platelet activation in response to collagen. The recognition sequence on collagen consists of (GlyProHyp) $_n$  sequences. Patients are known from Japan who have a genetic deficiency of GPVI. They have mild bleeding problems and their platelets respond only weakly to collagen, presumably via other receptors. A great deal has been learned about the signalling cascades originating at GPVI which strongly resemble those from immune receptors including T-cell receptors, B-cell receptors and natural killer cell receptors. These cascades involve src family tyrosine kinases such as Fyn and Lyn as well as p72<sup>SYK</sup> and many other tyrosine kinases and phosphatases and adaptor proteins such as LAT. A main target of these cascades is activation of phospholipase C $\gamma$ 2 which splits phospholipids to give the second messengers diacylglycerol and IP $_3$ . GPVI is thought to be involved in activation of the platelet integrin  $\alpha$ 2 $\beta$ 1 which has a major role in platelet adhesion to damaged vessel wall. Mice with the Fc $\gamma$  subunit "knocked-out" have platelets which still show responses to collagen implying that the resting state of  $\alpha$ 2 $\beta$ 1 may also be regulated by the GPVI/Fc $\gamma$  complex.

5

The platelet collagen receptor GPVI is closely related to the natural killer  
activatory receptors of the p58KAR family as well as to Fc $\alpha$ R.

10

15

20

25

30

35

40

45

50

55

The adhesion and activation of resting, circulating platelets at a site of vascular  
injury is the first step in a process leading to the formation of a thrombus which is  
converted into a haemostatic plug. Collagen is one of the major components of  
the vessel wall responsible for platelet activation. Many types of collagen exist  
and seven of these are found in the subendothelial layers. Several different  
receptors for collagen have been identified on platelets but the major ones are  
now considered to be the integrin  $\alpha_2\beta_1$  and the non-integrin GPVI. Although  $\alpha_2\beta_1$   
is well characterised and both subunits were cloned and sequenced several  
years ago, the structure of GPVI has remained elusive although several features  
have been identified. It was determined about twenty years ago that GPVI is a  
major platelet glycoprotein with a molecular mass in the 60-65 kDa range and an  
acid pI. Its role as a putative collagen receptor was established following the  
identification of a patient in Japan with a mild bleeding disorder whose platelets  
showed a specific defect of response to collagen and lacked this receptor. This  
patient had also developed autoantibodies to the deficient receptor and these  
were used to characterise the molecule further. More recently it was established  
that GPVI is associated non-covalently with the common Fc $\gamma$  subunit which acts  
as the signalling part of the complex. It was also demonstrated that the  
recognition sequence on collagen for GPVI is a repeated Gly-Pro-Hyp triplet  
within the collagen triple helical structure and that synthetic peptides based on  
this structure could be used as specific GPVI directed agonists. The GPVI/ Fc $\gamma$   
complex was shown to signal to the platelet interior by an immune receptor-like  
mechanism, involving activation of p72<sup>SYK</sup> and leading by a cascade of  
kinase/phosphatase/adaptor protein interactions to activation of PLC $\gamma$ 2 and  
hence to release of granules and platelet aggregation. A further step in  
characterisation of this molecule was the demonstration that the snake C-type  
lectin, convulxin, from the Tropical Rattlesnake, *Crotalus durissus terrificus* was  
able to activate platelets by clustering GPVI through a multimeric interaction.  
Convulxin was shown to bind specifically to GPVI providing a method for  
purification of this receptor in conjunction with established approaches.

5

10

15

20

25

30

35

40

45

50

55

Thus, it is clear from the prior art that GPVI seems to be a very interesting compound in many therapeutical fields above all concerning with applications which are related, directly or indirectly, to blood coagulation events which depend on collagen – platelet interaction. It was, therefore, the goal of the present invention is to provide GPVI in a recombinant form and to show its efficiency as direct therapeutical target or as tool for screening of short compounds, especially chemically synthesized or synthesizable compounds having the capability to inhibit or block the natural platelet-collagen interaction.

The invention relates also to portions or fragments of the GPVI protein which have maintained their biological activity which is the binding to collagen.

The invention was successful in purifying adequate amounts of GPVI for preliminary characterisation and for peptide sequencing. The sequences were used to design primers for PCR to identify a positive sequence in a DNA library. This DNA sequence was then used as a probe to isolate an almost complete cDNA sequence from the library and missing 5'-sequence was obtained using a RACE method from a platelet cDNA library.

The invention was also successful in showing the use of recombinant GPVI as therapeutically applicable compound which is capable, when administered in a patient with e.g. damaged blood vessels, to bind to collagen, thus preventing platelets bearing membrane-bound GPVI from binding to said collagen. The recombinant soluble extracellular domain of GPVI contains the collagen binding site and can prevent platelet activation by collagen. It could therefore be applicable to treatment of disease conditions involving increased platelet activation with collagen, such as atherosclerotic plaque rupture, in diseases such as unstable angina or, during surgical treatment such as Percutaneous Transluminal Coronary Angioplasty (PTCA), where arteries are reopened by inflation of a balloon catheter causing considerable damage to the vessel wall and much platelet activation and often resulting in reclosure of the vessel later. The advantage of recombinant GPVI fragments compared to present treatment methods is that they act at an earlier stage by preventing or reducing platelet activation rather than by suppressing events after platelet activation, such as

5 aggregation by GPIIb-IIIa antagonists. Thus, smaller amounts of platelet granule  
contents are released including growth factors and chemokines which are  
10 involved not only in wound repair but in the remodelling of the vessel wall by  
smooth muscle migration and in attraction of phagocytic cells such as monocytes  
5 known to contribute to atherosclerosis. Fab fragment of humanised mouse  
monoclonal antibodies against GPVI could be used with similar effect to block  
15 GPVI on the platelet surface with similar applications as above.

Recombinant GPVI according to this invention can also be used in a binding  
20 assay to collagen to screen for small molecules (in combinatorial libraries for  
example) capable of inhibiting this interaction and which can be used to develop  
therapeutic compounds which are inhibitors of the collagen-platelet interaction .  
By suitable derivatisation these compounds are made orally available. Again the  
25 main objective is to prepare compounds reducing GPVI-collagen interactions and  
hence platelet activation in situations where platelets come into contact with  
15 collagen. The screening technology as such used in this invention is well  
established in the prior art. By such screening assays the invention enables  
30 finding and developing new targets which can inhibit the natural membrane-  
bound GPVI on the platelet surface as a collagen antagonist. Such targets which  
20 may be small chemical molecules may then be the basis for further inventions.

35 Another major application of GPVI and reagents that recognize specific domains  
of GPVI is as markers of platelet age and functionality. Young platelets are  
generally thought to be more active and functional than older ones. Young  
40 platelets bind to and are activated by the snake venom C-type lectin convulxin,  
25 which is specific for GPVI, and as they age both the binding and degree of  
activation decrease. This can be due to either proteolytic or conformational  
changes in GPVI or its association with Fcγ due to platelet activation or damage  
45 in the circulation. This can be a useful parameter to measure the age and  
function profile of platelets in patients as well as in normal persons during medical  
30 controls. The platelet age profile changes in many diseases affecting the bone  
marrow or the immune system and could be an important diagnostic criterion if  
50 better methods for its determination were available. For example, patients with

5 diseases involving increased platelet turnover will show more young platelets  
whereas patients on chemotherapy or radiation treatment will show a steadily  
10 aging population. Thus, such an age profile can be used for a precise monitoring  
of treatment. In a normal healthy population very little is known about the age  
5 profile distribution and its role as a predictor of changes in health. It is not known  
whether the changes in GPVI are due to the partial involvement of platelets in  
15 haemostatic events and whether changes might be more pronounced in patients  
with extensive cardiovascular disease. At present thiazole orange is used to  
detect young reticulated platelets containing mRNA. This mRNA soon decays,  
10 restricting the method to only the youngest platelets. Reagents which could be  
used in such an assay would include GPVI-specific snake venom proteins such  
as convulxin, or monoclonal or polyclonal antibodies recognising the N-terminal  
region of GPVI or monoclonal antibodies recognising new sites or conformations  
25 exposed by proteolysis of the N-terminal domain or specific conformations  
present either in the intact molecule and not in the aged one or vice versa or  
small chemical entities selected to recognise specifically intact GPVI or its  
30 modified form. These reagents would be labelled with a fluorescent marker, or  
together with a fluorescent labelled second antibody or affinity reagent and used  
in flow cytometry to measure the platelet binding profile. At a later stage  
20 alternative, less labour intensive measuring techniques based on automated  
measuring of platelet profiles could be adopted. Using cell sorting methods with  
35 flow cytometry or magnetic beads it should be possible to isolate young and old  
platelets to examine the factors involved in removal of old platelets from the  
circulation. Reagents recognizing specific forms of GPVI would be a key to such  
40 studies.

45 Therefore, it is an object of the present invention to provide a DNA coding for  
Glycoprotein VI or biological active fragments thereof, especially the sequence of  
Fig. 2.

50 It is a further object of this invention to provide a DNA coding for Glycoprotein VI  
comprising the amino acid sequences of Fig. 1a and 1b.

5

10

It is another object of this invention to provide a pharmaceutical composition comprising recombinant GPVI together with a pharmaceutically acceptable diluent, carrier or excipient, and its use for the manufacture of a medicament in the therapeutical field of thrombotic and cardiovascular events and disorders related to platelet-collagen interactions

15

Another object of the invention is the use of recombinant GPVI in a screening tool for detecting specific inhibitors of platelet-collagen interactions.

Another object of the invention is the use of GPVI as a marker for platelet age and exposure to cardiovascular diseases.

20

10

25

15

Possible medical indications and applications, respectively, are, for example, unstable angina pectoris, PTCA, use of stents in this field, operations on coronary vessels, general operations on blood vessels, operations which may damage larger blood vessels such as hip joint operations. Moreover, all indications are included which relate to thromboembolic events caused by disorders of the interaction between the vessel wall and the coagulation system with a high risk of formation of thrombi and blocking of vessels.

30

35

As indicated above, the GPVI protein and fragments thereof according to the present invention are suitable as pharmaceutically effective compounds in pharmaceutical compositions and combinations.

40

25

The pharmaceutical formulations according to the invention optionally may comprise additional active ingredients like anti-coagulants such as hirudin or heparin or thrombolytic agents such as plasminogen activator or hementin or antagonists to other platelet receptors such as GPIIb-IIIa antagonists like abciximab or eptifibatide or ADP-receptor antagonists such as clopidogrel.

45

50

The novel protein, and its biological active fragments respectively, according to the invention may form pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Inorganic acids are, for example, hydrochloric, hydrobromic, sulphuric or phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Examples for

55

5 organic acids are the mono, di and tri carboxylic acids such as acetic, glycolic,  
lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic,  
10 maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic  
and sulfonic acids such as methane sulfonic acid. Salts of the carboxy terminal  
5 amino acid moiety include the non-toxic carboxylic acid salts formed with any  
suitable inorganic or organic bases. These salts include, for example, alkali  
15 metals such as sodium and potassium, alkaline earth metals such as calcium and  
magnesium, light metals of Group IIIA including aluminium, and organic primary,  
secondary and tertiary amines such as trialkylamines, including triethylamine,  
20 procaine, dibenzylamine, 1-ethenamine, N,N'-dibenzylethylene-diamine,  
dihydroabietylamine and N-alkylpiperidine.

25 As used herein, the term "pharmaceutically acceptable carrier" means an inert,  
non toxic solid or liquid filler, diluent or encapsulating material, not reacting  
15 adversely with the active compound or with the patient. Suitable, preferably liquid  
carriers are well known in the art such as sterile water, saline, aqueous dextrose,  
30 sugar solutions, ethanol, glycols and oils, including those of petroleum, animal,  
vegetable, or synthetic origin, for example, peanut oil, soybean oil and mineral oil.

20 The formulations according to the invention may be administered as unit doses  
35 containing conventional non-toxic pharmaceutically acceptable carriers, diluents,  
adjuvants and vehicles which are typical for parenteral administration.

40 The term "parenteral" includes herein subcutaneous, intravenous, intra-articular  
25 and intratracheal injection and infusion techniques. Also other administrations  
such as oral administration and topical application are suitable. Parenteral  
compositions and combinations are most preferably administered intravenously  
45 either in a bolus form or as a constant fusion according to known procedures.  
Tablets and capsules for oral administration contain conventional excipients such  
30 as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and  
wetting agents. The tablets may be coated according to methods well known in  
50 the art.

5

10

15

20

25

30

35

40

45

50

55

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives like suspending agents, emulsifying agents, non-aqueous vehicles and preservatives. Topical applications may be in the form of aqueous or oily suspensions, solutions, emulsions, jellies or preferably emulsion ointments.

Unit doses according to the invention may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, sex, diet, time and route of administration, rate of clearance, the object of the treatment, i.e., therapy or prophylaxis and the nature of the thrombotic disease to be treated, antiplatelet or anticoagulant activity.

Therefore, in compositions and combinations useful as anticoagulants in a treated patient (in vivo) a pharmaceutical effective daily dose of the peptides of this invention is between about 0.01 and 100 mg/kg body weight, preferably between 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of the collagen inhibitor To achieve an anticoagulant effect in extracorporeal blood a pharmaceutically effective amount of the inventive peptides is between 0.2 and 150 mg/l, preferably between 1mg and 20 mg/l extracorporeal blood.

Short Description of the Figures:

Fig. 1: Protein sequence of GPVI (one-letter-code)

1a: Leader sequence

1b: Mature protein

*Open reading frame: 339 amino acids*

*Asterisk:* Glycosylation site  
*Double underline:* Transmembrane domain  
*Underline:* Sequenced peptides

Fig. 2: GPVI nucleotide sequence covering open reading frame of 1017 bp plus 3' and 5' regions total 1249 bp

Detailed description of the invention

Two sequences of 7 amino acids showing the least degeneracy in the genetic code were chosen for the synthesis of DNA primers in order to amplify part of the of GPVI cDNA by PCR. As the location of both peptides in the protein were totally unknown, for each of them, two degenerate primers, one sense and one antisense were prepared. These primers were used to amplify a human bone-marrow library. The combination of the sense 5' TYA THC CNG CNA TGA ARMG 3' primer coding for the sequence PAMKRSL with the antisense 5' TTR TAN ARN GCR AAY TGR TC 3' one corresponding to DQFALYK amplified a DNA fragment of 221 bp. In addition to the selected peptides, the amplified DNA coded for the LysC/AspN peptide DQLELVATGVFAKPSLSAQPGPAVSS, clearly linking the sequence to the cDNA for GPVI.

Screening 600.000 pfu from a bone marrow library with this 221 bp DNA fragment produced 4 positive pfu. Three had inserts of 1350 bp whether cut by the restriction enzymes Sal I or EcoR I and belonged to the IgG superfamily. The fourth one had an 4.6 kb insert by Sal I digestion and gave two fragments of 2300 bp and 1300 bp respectively when treated by EcoRI. Its DNA encoded the sequence for the 10 peptides derived from amino acid sequencing of GPVI but stopped short of the amino terminal. No starting methionine or leader sequence could be found but more than 2000 bp of previously sequenced non-reading frame DNA terminating in an Alu sequence were present. The 5' end RACE experiment was completed on platelet poly A RNA with primers located in a part of the GPVI sequence which had been corroborated by that of the peptides. A fragment of 348 bp including 278 bp on the sequence of the fourth clone and 70 bp new from bp 1987 corresponding to 14 amino acids including the first

5

10

methionine were found before falling back on the established GPVI sequence. Thus, a cDNA containing a total of 1249 bp, a 25 bp 5' sequence upstream of the start codon, an open reading frame of 1017 bp coding for a protein, including leader sequence, with 339 amino acids, and a 3' region of 207 bp including the stop codon could be sequenced.

15

20

25

30

35

40

45

50

55

A cDNA coding for platelet GPVI was cloned and sequenced from a human bone marrow cDNA library using RACE with platelet mRNA to supply missing 5' sequence. The open reading frame of 1017 bp encodes 339 amino acids and a untranslated 3' region. Hydrophobicity analysis of the amino acid sequence revealed the presence of two putative transmembrane domains, a putative 20 amino acid signal sequence, and a 19 amino acid domain between residues 247 and 265 of the mature protein. The sequence and its amino acid translation are shown in Fig. 2 and Fig. 1. A comparison with the amino acid sequence of the most similar molecules found in a search of GenBank reveals clearly that it belongs to the immunoglobulin superfamily and the extracellular domain contains two Ig C2-domain loops formed by two disulphide bridges. It is a membrane crossing protein class one molecule with the N-terminus at the exterior and traverses the membrane once. The most closely related molecules belong to the natural killer receptor class which contains both inhibitory and activatory types. GPVI clearly belongs to the activatory subclass not only through its function but also because unlike the inhibitory class it does not contain ITIM sequences in its cytoplasmic domain. Neither does it contain any tyrosine residues which might be involved in phosphorylation. There are some threonine and serine residues in this domain but they do not match any criteria for kinase consensus sequences. Like the activatory class of NK receptors, GPVI contains an arginine residue as the third amino acid of the membrane crossing domain which is involved in the complex formation with the Fc $\gamma$  subunit. The cytoplasmic domain contains 51 amino acids, showing only a minor similarity (in the region just below the membrane) to the cytoplasmic domains of other members of this family. This suggests that this domain in GPVI may associate with different types of cytoplasmic molecule than the other family members. GPVI contains only a single putative N-glycosylation site at Asn69. The domain just above the membrane

5

10

15

20

25

30

35

40

45

50

55

after the beta sheets of the Ig domains finish, however, is rich in theonine and serine residues which could provide O-glycosylation sites such as are found in GPIIb $\alpha$  and GPV. The main function of this O-glycosylation seems to be to present the receptor structures well-extended from the platelet surface to facilitate the interactions with their bulky ligands. Since GPVI was earlier established as a sialoglycoprotein, the difference in molecular mass between the theoretical amino acid mass (37 kDa) and the mass determined by gel electrophoresis (65 kDa reduced) must be due to this glycosylation.

- 10 The structure of natural killer receptors of the two domain type has been established by X-ray crystallographic studies and the two Ig-domains were shown to form an acute angle with the receptor site for the peptide-carrying HLA antigens lying on the outside of the elbow. A direct comparison of the structure of the HLA peptide binding site with that of collagen immediately suggests why these receptors have a common origin because the multiple alpha-helical structures of the HLA binding site and the peptide it contains strongly resemble the triple helical structure of collagen. The natural killer receptors are postulated to work by a dimerisation mechanism with two receptors recognising two separate HLA sites on the cell which the natural killer cell is investigating. Possibly this dimerisation is part of the activation or deactivation mechanism, depending on the class of receptor. In the case of GPVI there may as well be the possibility for two GPVI molecules to associate with one Fc $\gamma$ , since each monomer of the Fc $\gamma$  dimer has a recognition sequence. However, the stoichiometry is not yet known, and based upon the structure of collagens, collagen-like peptides that act via GPVI and convulxin, it seems likely that the strength of the signal is related to the number of GPVI/Fc $\gamma$  complexes that are clustered together. Other platelet receptors belonging to this Ig family include ICAM-2 (CD102 )and PECAM (CD31).

- 30 All microorganisms, cell lines, expression systems, expression hosts, plasmids, promoters, resistance markers, replication origins, restriction sites or other fragments or parts of vectors which are mentioned in the description not directly in connection with the claimed invention are commercially or otherwise generally

5 available. Provided that no other hints are given, they are used only as examples  
and are not essential with respect to the invention, and can be replaced by other  
10 suitable tools and biological materials, respectively.

- 5 The techniques which are essential according to the invention are described in  
detail below and above. Other techniques which are not described in detail  
15 correspond to known standard methods which are well known to a person skilled  
in the art, or are described more in detail in the cited references and patent  
applications and in the standard literature (e.g. Sambrook et al., 1989, Molecular  
10 Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor; Harlow, Lane,  
20 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor).

#### EXAMPLES

- 25  
15 Example 1: *Materials*—Protein A-Sepharose, peroxidase-conjugated goat anti-  
mouse and anti-rabbit antibodies, bovine serum albumin, *Crotalus durissus*  
30 *terrificus* venom, wheat germ agglutinin (WGA), N-hydroxysuccinimidyl  
chloroformate-activated cross-linked 4% beaded agarose and Triton X-114 were  
from Sigma Chemical Co. (St Louis, MO), Octanoyl-N-methyl-glucamide (ONMG)  
20 and nonanoyl-N-methyl-glucamide (NNMG) were from OxyL Chemie (Bobingen,  
35 Germany).

- Example 2: *GPVI isolation from platelets* — Membrane glycoproteins were  
40 isolated from platelets as previously described. Briefly, platelets (from 40 buffy  
25 coats) were washed and lysed in 2% Triton X-114 in the presence of protease  
inhibitors. The Triton X-114 and aqueous phases were separated and the  
detergent phase was loaded on a column of wheat-germ agglutinin coupled to  
45 Sepharose 4B. The platelet glycoproteins were eluted with 10 mM Tris HCl, pH  
7.4, 30 mM NaCl, 0.2% octanoyl-N-methylglucamide (ONMG) and 2% N-  
30 acetylglucosamine. After dialysis and concentration, the solution of glycoproteins  
was loaded on a column of convulxin bound to N-hydroxysuccinamidyl-p-  
50 nitrophenyl chloroformate activated cross-linked 4% beaded agarose (1 mg/ml).  
The column was washed with 4 volumes of 10 mM Tris HCl, pH 7.4, 30 mM NaCl,

0.2% nonanoyl-N-methylglucamide (NNMG), and then with 4 volumes of 10 mM Tris HCl, pH7.4, 30 mM NaCl and 2% NNMG. GPVI was eluted with 0.08% SDS in 10 mM Tris/HCl, pH 7.4. The solution was concentrated and loaded on a preparative gel of 8.5 % polyacrylamide using the Model 491 Prep Cell (BioRad, CA). The preparative electrophoresis was performed under non-reduced conditions following the manufacturer instructions. GPVI eluted as a single band at 65 kDa. The fractions were pooled, concentrated on Centricon-30 (Amicon, Beverly, MA) and resuspended in 10 mM Tris/HCl, pH7.4 and 0.1% ONMG.

**Example 3:** *Amino acid analysis of GPVI* - GPVI was digested with the endoproteinases LysC and AspN (Boehringer Mannheim, Germany). The 10 peptides generated were separated by reverse-phase HPLC and sequenced on an Applied Biosystem model 477A pulsed-liquid-phase protein sequencer with a model 120A on-line phenylthiohydantoin amino acid analyser.

**Example 4:** *Amplification of a 221 bp fragment coding for part of GPVI from a  $\lambda$ gt11 cDNA library* -

A sample ( $10^{10}$  pfu) (plaque forming units) from a human bone marrow library (Clontech, Palo Alto, CA) was amplified using 2 combinations of 4 degenerate primers. The final primer concentrations were 2  $\mu$ M, the dNTP concentration was 200  $\mu$ M and 2 U/100  $\mu$ l reaction AmpliTaq Gold (Perkin Elmer, Rotkreuz, Switzerland) were used. The PCR conditions were 5 cycles at 37°C followed by 30 cycles at 44°C. The sense 19mer 5' TYATHCCNGCNATGAARMG 3' and the antisense 20mer 5' TTRTANARNGCRAAYTGRTC 3' amplified a 221 bp fragment which was subcloned in Bluescript KS<sup>+</sup> (Stratagene, La Jolla, CA) and sequenced using the T7 Sequenase kit (Amersham, Switzerland).

**Example 5:** *Screening the  $\lambda$ gt11 cDNA library with the 221 bp GPVI probe* - The 221 bp fragment was cut from the plasmid, cleaned and labelled with  $\alpha^{32}$ P-ATP (20MBq/50  $\mu$ l, Hartmann Analytik, Braunschweig, Germany) using the High Prime Labelling kit (Boehringer Mannheim, Switzerland). The human bone marrow library was screened following the manufacturer instructions. Positive phages were grown, their DNA isolated and subcloned in BlueScript using either EcoRI or

5 Sal I sites and sequenced. Sequencing was performed using the ABS system of  
RACE- Platelet poly A RNA was prepared as previously described (Power et al.,  
10 Cytokine 7, 479-482, 1995). Reverse transcription (30 µl) was performed using 5  
µg of poly A RNA with the primer 5'TGAATGAGACGGTCAGTTCAGC 3' (20 µM),  
5 dNTP (1mM), RNAsin (40 U), 1X AMV buffer and 20 U AMV reverse  
transcriptase for 20 min at 45°C followed by 20 min at 52°C. The reaction mixture  
15 was treated with 2 µl 6N NaOH at 65° C for 30 min, neutralised with 2µl 6N acetic  
acid, and concentrated in a Centricon 30 (Amicon). An anchor was ligated to the  
first strand DNA following the protocol of Aptes and Siebert (BioTechniques 15:  
20 890-893, 1993). Nested PCR was performed using a primer complementary to  
the anchor and the primer 5' TTGTACAGAGCAAATTGGTC 3' (35 cycles, 55°C)  
and followed by the primer 5' GACCAGAGGCTTCCGTTCTG 3' (30 cycles at  
25 53°C). The highest band (350 bp) was separated by agarose electrophoresis  
from the lower ones, subcloned into BlueScript, and sequenced.

15 Example 6: Preparation of anti-GPVI Fab and F(ab')<sub>2</sub>-Polyclonal antisera  
30 against human GPVI were generated in rabbits. IgG from rabbit anti-GPVI  
antiserum was purified as described. Digestion of IgG with immobilized papain  
(Pierce) to generate Fab fragments was performed according to the standard  
20 protocol of the supplier. Fab fragments were separated from undigested IgG and  
Fc fragments using an immobilized Protein A (Sigma) column. The flowthrough  
35 was transferred to a dialysis tube, concentrated using solid polyethyleneglycol  
20,000, thoroughly dialysed against 20 mM Hepes, 140 mM NaCl, 4 mM KCl, pH  
40 7.4 and stored at 4°C until used. F(ab')<sub>2</sub> fragments were prepared by pepsin  
25 digestion of IgG, 1:50 enzyme to substrate ratio (w/w), in 0.5 M acetate buffer, pH  
4.0, at 37°C for 18 hr. The pH was corrected to 7.4 with diluted NaOH and the  
45 sample was dialysed against 20 mM phosphate, pH 7.4. F(ab')<sub>2</sub> fragments were  
separated from undigested IgG and Fc fragments using Protein A  
chromatography. The flow-through was transferred to dialysis tube, concentrated  
50 using solid polyethyleneglycol 20 000, intensively dialysed against 20 mM Hepes,  
140 mM NaCl, 4 mM KCl, pH 7.4 and stored in aliquots at -20°C. Washed  
platelets were lysed in Triton X-114 and phase separation was performed on the  
55

soluble material before isolating the membrane glycoproteins associated with the Triton X-114 phase by affinity chromatography on wheat germ agglutinin-Sepharose 4B as described previously. As GPVI represents a very small fraction of the platelet membrane glycoprotein pool, we used the specificity of the snake C-type lectin convulxin for isolation of this receptor. Affinity chromatography on convulxin coupled to Sepharose 4B yielded a 65 kDa protein as major product. However, uncharacterized material of both higher and lower Mr co-eluted with GPVI and could not be removed by extensive washing of the column. Preparative gel electrophoresis on 8.5 % polyacrylamide was added as a final step of purification. Fractions containing GPVI were pooled and gave a single band on reanalysis. Purified GPVI was tested for its ability to block platelet aggregation by collagen. A slight inhibitory effect was observed when aliquots of GPVI solution were added to the platelet suspension. However, by preincubating GPVI with collagen before adding the mixture to the platelet suspension, aggregation could be inhibited in a dose-dependant manner. These platelets still aggregated when fresh collagen was added. Under non-reducing conditions, the isolated protein has a Mr of 62 kDa with a shift toward a slightly higher Mr (65 kDa) under reducing conditions. As the amino terminus of GPVI was found to be blocked, the protein was digested with the enzymes LysC and LysC/AspN which produced 4 and 6 peptides, respectively, from which sequence was obtained. The peptides were separated by reverse phase HPLC on a C4 column and sequenced using the Edman method. The amino acid sequences of these peptides are underlined in the translated cDNA sequence (Fig. 1).

## Claims

5

10

15

20

25

30

35

40

45

50

55

5

**Patent claims**

10

1. DNA coding for Glycoprotein VI or biologically active fragments thereof.

5

15

2. DNA according to claim 1 comprising partially or completely the sequence of Fig. 2.

20

3. DNA having the sequence of Fig. 2

10

4. DNA according to claims 1 – 3 coding for Glycoprotein VI comprising the amino acid sequence of Fig. 1a and 1b.

25

5. Recombinant human Glycoprotein VI as medicament comprising the amino acid sequence of Fig. 1b.

15

30

6. Pharmaceutical composition comprising the protein of claim 5 and a pharmaceutically acceptable diluent, carrier or excipient therefor.

35

7. Pharmaceutical composition comprising additionally a pharmacologically active compound.

40

8. Use of recombinant GPVI in a screening tool for detecting specific inhibitors of platelet-collagen interactions.

25

45

9. Use of recombinant GPVI for the manufacture of a medicament in the therapeutical field of thrombotic and cardiovascular events and disorders related to platelet-collagen interactions.

50

10. Use of changes in GPVI as a marker for platelet age and platelet exposure to thrombotic and cardiovascular conditions or events.

55

**Figure 1.****(1a)**

1 20  
MSPSP TALFC LGLCLGRVPA

**(1b)**

1  
QSGPLPKPSL QALPSSLVPL EKPVTLCQG PPGVDLYRLE KLSSRYQDO (50)  
AVLFIPAMKR SLAGRYRCSY QN\*GSLWSLPS DQLELVATGV FAKPSLSAQP (100)  
GPAVSSGGDV TLCQCTRYGF DQFALYKEGD PAPYKNPERW YRASFPITV (150)  
TAAHSGTYRC YSFSSRDPLY WSAPSDPLEL VVTGTSVTPS RLPTEPPSSV (200)  
AEFSEATAEL TVSFTNKVET TETSRISITS PKESDSPAGP ARQYYTKGNL (250)  
VRICLGAVIL ILLAGFLAED WHSRKRLRH RGRAVQRPLP PLPPLPQTRK (300)  
SHGGQDGGGRQ DVHSRGLCS (319)

Figure 2:

GAGCTCAGGA CAGGGCTGAG GAACCATGTC TCCATCCCCG ACCGCCCTCT (50)  
TCTGTCTTGG GCTGTGTCTG GGGCGTGTGC CAGCGCAGAG TGGACCGCTC (100)  
CCCAAGCCCT CCCTCCAGGC TCTGCCCAGC TCCCTGGTGC CCCTGGAGAA  
GCCAGTGACC CTCCGGTGCC AGGGACCTCC GGGCGTGGAC CTGTACCGCC (200)  
TGGAGAAGCT GAGTTCAGC AGGTACCAGG ATCAGGCAGT CCTCTTCATC  
CCGGCCATGA AGAGAAGTCT GGCTGGACGC TACCGCTGCT CCTACCAGAA (300)  
CGGAAGCCTC TGGTCCCTGC CCAGCGACCA GCTGGAGCTC GTTGCCACGG  
GAGTTTTTGC CAAACCCTCG CTCTCAGCCC AGCCCCGCCC GCGGGTGTGC (400)  
TCAGGAGGGG ACGTAACCCT ACAGTGTGAG ACTCGGTATG GCTTTGACCA  
ATTGTCTCTG TACAAGGAAG GGGACCCTGC GCCCTACAAG AATCCCGAGA (500)  
GATGGTACCG GGCTAGTTTCC CATCATCA CGGTGACCGC CGCCACAGC  
GGAACCTACC GATGCTACAG CTTCTCCAGC AGGGACCCAT ACCTGTGGTC (600)  
GGCCCCCAGC GACCCCTGG AGCTTGTGGT CACAGGAACC TCTGTGACCC  
CCAGCCGGTT ACCAACAGAA CCACCTCCT CGGTAGCAGA ATTCTCAGAA (700)  
GCCACCGCTG AACTGACCGT CTCATTACA AACAAAGTCT TCACAAGTGA  
GACTTCTAGG AGTATCAGCA CCAGTCCAAA GAGTCAGAC TCTCCAGCTG (800)  
GTCTGCCCCG CCAGTACTAC ACCAAGGGCA ACCTGGTCCG GATATGCCTC  
GGGGCTGTGA TCCTAATAAT CCTGGCGGGG TTTCTGGCAG AGGACTGGCA (900)  
CAGCCGGAGG AAGCGCTGC GGCACAGGGG CAGGGCTGTG CAGAGGCCGC  
TTCCGCCCTT GCGCCCTC CCGCAGACCC GGAATCACA CGGGGGTCAG (1000)  
GATGGAGGCC GACAGGATGT TCACAGCCGC GGGTTATGTT CATGACCGCT  
GAACCCAGG CACGGTCGTA TCCAAGGGAG GGATCATGGC ATGGGAGGCG (1100)  
ACTCAAAGAC TGGCGTGTGT GGAGCGTGA AGCAGGAGGG CAGAGGCTAC  
AGCTGTGAA ACGAGGCCAT GCTGCCTCCT CCTGGTGTT CATCAGGGAG (1200)  
CCGTTCGGCC AGTGTCTGTC TGCTGTCTG CCTCTCTGTC TGAGGGCAC (1249)

SEQUENCE LISTING

<110> Merck Patent GmbH

5 <120> Recombinant platelet collagen receptor glycoprotein VI  
and its pharmaceutical use

<130> GlycoproteinVI-Merck

10 <140>  
<141>  
<160> 1

15 <170> PatentIn Ver. 2.1

<210> 1  
<211> 1249  
<212> DNA

20 <213> Homo sapiens

<220>  
<221> CDS  
<222> (26)..(85)

25 <223> Leader sequence

<220>  
<221> CDS  
<222> (86)..(1042)

30 <223> mature GPVI protein

<400> 1  
gagctcagga cagggctgag gaacc atg tct cca tcc ccg acc gcc ctc ttc 52  
Met Ser Pro Ser Pro Thr Ala Leu Phe  
1 5

35 tgt ctt ggg ctg tgt ctg ggg cgt gtc cca gcg cag agt gga ccg ctc 100  
Cys Leu Gly Leu Cys Leu Gly Arg Val Pro Ala Gln Ser Gly Pro Leu  
10 15 20 25

40 ccc aag ccc tcc ctc cag gct ctg ccc agc tcc ctg gtg ccc ctg gag 148  
Pro Lys Pro Ser Leu Gln Ala Leu Pro Ser Ser Leu Val Pro Leu Glu  
30 35 40

45 aag cca gtg acc ctc cgg tgc cag gga cct ccg ggc gtg gac ctg tac 196  
Lys Pro Val Thr Leu Arg Cys Gln Gly Pro Pro Gly Val Asp Leu Tyr  
45 50 55

50 cgc ctg gag aag ctg agt tcc agc agg tac cag gat cag gca gtc ctc 244  
Arg Leu Glu Lys Leu Ser Ser Ser Arg Tyr Gln Asp Gln Ala Val Leu  
60 65 70

55 ttc atc ccg gcc atg aag aga agt ctg gct gga cgc tac cgc tgc tcc 292  
Phe Ile Pro Ala Met Lys Arg Ser Leu Ala Gly Arg Tyr Arg Cys Ser  
75 80 85

60 tac cag aac gga agc ctc tgg tcc ctg ccc agc gac cag ctg gag ctc 340  
Tyr Gln Asn Gly Ser Leu Trp Ser Leu Pro Ser Asp Gln Leu Glu Leu  
90 95 100 105

gtt gcc acg gga gtt ttt gcc aaa ccc tcg ctc tca gcc cag ccc ggc 388  
 Val Ala Thr Gly Val Phe Ala Lys Pro Ser Leu Ser Ala Gln Pro Gly  
 110 115 120

5 ccg gcg gtg tcg tca gga ggg gac gta acc cta cag tgt cag act cgg 436  
 Pro Ala Val Ser Ser Gly Gly Asp Val Thr Leu Gln Cys Gln Thr Arg  
 125 130 135

10 tat gcc ttt gac caa ttt gct ctg tac aag gaa ggg gac cct gcg ccc 484  
 Tyr Gly Phe Asp Gln Phe Ala Leu Tyr Lys Glu Gly Asp Pro Ala Pro  
 140 145 150

15 tac aag aat ccc gag aga tgg tac cgg gct agt ttc ccc atc atc acg 532  
 Tyr Lys Asn Pro Glu Arg Trp Tyr Arg Ala Ser Phe Pro Ile Ile Thr  
 155 160 165

20 gtg acc gcc gcc cac agc gga acc tac cga tgc tac agc ttc tcc agc 580  
 Val Thr Ala Ala His Ser Gly Thr Tyr Arg Cys Tyr Ser Phe Ser Ser  
 170 175 180 185

25 agg gac cca tac ctg tgg tcg gcc ccc agc gac ccc ctg gag ctt gtg 628  
 Arg Asp Pro Tyr Leu Trp Ser Ala Pro Ser Asp Pro Leu Glu Leu Val  
 190 195 200

gtc aca gga acc tct gtg acc ccc agc cgg tta cca aca gaa cca cct 676  
 Val Thr Gly Thr Ser Val Thr Pro Ser Arg Leu Pro Thr Glu Pro Pro  
 205 210 215

30 tcc tcg gta gca gaa ttc tca gaa gcc acc gct gaa ctg acc gtc tca 724  
 Ser Ser Val Ala Glu Phe Ser Glu Ala Thr Ala Glu Leu Thr Val Ser  
 220 225 230

35 ttc aca aac aaa gtc ttc aca act gag act tct agg agt atc acc acc 772  
 Phe Thr Asn Lys Val Phe Thr Thr Glu Thr Ser Arg Ser Ile Thr Thr  
 235 240 245

40 agt cca aag gag tca gac tct cca gct ggt cct gcc cgc cag tac tac 820  
 Ser Pro Lys Glu Ser Asp Ser Pro Ala Gly Pro Ala Arg Gln Tyr Tyr  
 250 255 260 265

45 acc aag gcc aac ctg gtc cgg ata tgc ctc ggg gct gtg atc cta ata 868  
 Thr Lys Gly Asn Leu Val Arg Ile Cys Leu Gly Ala Val Ile Leu Ile  
 270 275 290

atc ctg gcg ggg ttt ctg gca gag gac tgg cac agc cgg agg aag cgc 916  
 Ile Leu Ala Gly Phe Leu Ala Glu Asp Trp His Ser Arg Arg Lys Arg  
 285 290 295

50 ctg cgg cac agg gcc agg gct gtg cag agg ccg ctt ccg ccc ctg ccg 964  
 Leu Arg His Arg Gly Arg Ala Val Gln Arg Pro Leu Pro Pro Leu Pro  
 300 305 310

55 ccc ctc ccg cag acc cgg aaa tca cac ggg ggt cag gat gga gcc cga 1012  
 Pro Leu Pro Gln Thr Arg Lys Ser His Gly Gly Gln Asp Gly Gly Arg  
 315 320 325

60 cag gat gtt cac agc cgc ggg tta tgt tca tgaccgctga accccaggca 1062  
 Gln Asp Val His Ser Arg Gly Leu Cys Ser  
 330 335

cggtcgatc caagggagg atcatggcat gggaggcgac tcaaagactg gcgtgtgttg 1122  
 agcgtggaag caggaggga gaggtacag ctgtggaac gaggccatgc tgcctctcc 1182  
 5 tgggtgtcca tcaggagacc gtccggccag tgtctgtctg tctgtctgcc tctctgtctg 1242  
 agggcac 1249

10 <210> 2  
 <211> 20  
 <212> PRT  
 <213> Homo sapiens

15 <400> 2  
 Met Ser Pro Ser Pro Thr Ala Leu Phe Cys Leu Gly Leu Cys Leu Gly  
 1 5 10 15  
 Arg Val Pro Ala  
 20 20

<210> 3  
 <211> 319  
 25 <212> PRT  
 <213> Homo sapiens

<400> 3  
 Gln Ser Gly Pro Leu Pro Lys Pro Ser Leu Gln Ala Leu Pro Ser Ser  
 30 1 5 10 15  
 Leu Val Pro Leu Glu Lys Pro Val Thr Leu Arg Cys Gln Gly Pro Pro  
 20 25 30  
 35 Gly Val Asp Leu Tyr Arg Leu Glu Lys Leu Ser Ser Ser Arg Tyr Gln  
 35 40 45  
 Asp Gln Ala Val Leu Phe Ile Pro Ala Met Lys Arg Ser Leu Ala Gly  
 50 55 60  
 40 Arg Tyr Arg Cys Ser Tyr Gln Asn Gly Ser Leu Trp Ser Leu Pro Ser  
 65 70 75 80  
 Asp Gln Leu Glu Leu Val Ala Thr Gly Val Phe Ala Lys Pro Ser Leu  
 45 85 90 95  
 Ser Ala Gln Pro Gly Pro Ala Val Ser Ser Gly Gly Asp Val Thr Leu  
 100 105 110  
 50 Gln Cys Gln Thr Arg Tyr Gly Phe Asp Gln Phe Ala Leu Tyr Lys Glu  
 115 120 125  
 Gly Asp Pro Ala Pro Tyr Lys Asn Pro Glu Arg Trp Tyr Arg Ala Ser  
 130 135 140  
 55 Phe Pro Ile Ile Thr Val Thr Ala Ala His Ser Gly Thr Tyr Arg Cys  
 145 150 155 160  
 Tyr Ser Phe Ser Ser Arg Asp Pro Tyr Leu Trp Ser Ala Pro Ser Asp  
 60 165 170 175

Pro Leu Glu Leu Val Val Thr Gly Thr Ser Val Thr Pro Ser Arg Leu  
 180 185 190  
 5 Pro Thr Glu Pro Pro Ser Ser Val Ala Glu Phe Ser Glu Ala Thr Ala  
 195 200 205  
 Glu Leu Thr Val Ser Phe Thr Asn Lys Val Phe Thr Thr Glu Thr Ser  
 210 215 220  
 10 Arg Ser Ile Thr Thr Ser Pro Lys Glu Ser Asp Ser Pro Ala Gly Pro  
 225 230 235 240  
 Ala Arg Gln Tyr Tyr Thr Lys Gly Asn Leu Val Arg Ile Cys Leu Gly  
 245 250 255  
 15 Ala Val Ile Leu Ile Ile Leu Ala Gly Phe Leu Ala Glu Asp Trp His  
 260 265 270  
 Ser Arg Arg Lys Arg Leu Arg His Arg Gly Arg Ala Val Gln Arg Pro  
 275 280 285  
 20 Leu Pro Pro Leu Pro Pro Leu Pro Gln Thr Arg Lys Ser His Gly Gly  
 290 295 300  
 25 Gln Asp Gly Gly Arg Gln Asp Val His Ser Arg Gly Leu Cys Ser  
 305 310 315

&lt;160&gt; 3

30

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/EP 00/03683

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/705 A61K38/17 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, STRAND, WPI Data, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GIBBINS JONATHAN M ET AL: "Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain." FEBS LETTERS, vol. 413, no. 2, 1997, pages 255-259, XP002143941 ISSN: 0014-5793 the whole document	1
A	---	2-9
	---	-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document relating to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

Date of the actual completion of the international search

1 August 2000

Date of mailing of the international search report

21/08/2000

Name and mailing address of the ISA  
European Patent Office, P.B. 5616 Patentplan 2  
NL - 2200 HV Rijswijk  
Tel: (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/EP 00/03683

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JANDROT-PERRUS MARTINE ET AL: "Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha-2-beta-1." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 43, 1997, pages 27035-27041, XP002143942 ISSN: 0021-9258 the whole document	1
A	---	2-9
X	GIBBINS JONATHAN M ET AL: "The p85 subunit of phosphatidylinositol 3-kinase associated with the Fc receptor gamma-chain and linker for activator of T cells (LAT) in platelets stimulated by collagen and convulxin." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 51, 18 December 1998 (1998-12-18), pages 34437-34443, XP002143943 ISSN: 0021-9258 the whole document	1
A	---	2-9
L	WO 95 11259 A (THROMBOSIS RES INST ;JEHANLI AHMED MOHAMMED TAKI (GB); PATEL GEETA) 27 April 1995 (1995-04-27) abstract; claims 1-25	1
P,X	CLEMETSON JEANNINE M ET AL: "The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 41, 8 October 1999 (1999-10-08), pages 29019-29024, XP002143944 ISSN: 0021-9258 the whole document	1-5
P,X	MIURA Y: "Platelet glycoprotein VI" EMBL DATABASE ; ACCESSION NUMBER AB035073, 14 January 2000 (2000-01-14), XP002143945 the whole document	1-5

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No  
PCT/EP 00/03683

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9511259 A	27-04-1995	AU 701576 B	04-02-1999
		AU 7946594 A	08-05-1995
		EP 0724599 A	07-08-1996
		JP 9506863 T	08-07-1997
		NZ 275492 A	27-05-1998
		US 6001802 A	14-12-1999
		US 5952304 A	14-09-1999